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(54) Title: FOOD PRODUCT AND PROCESS FOR THEIR PREPARATION BY ENZYMATIC GALACTOSYLTRANSFER WITH LACTOBACILLUS ALPHA-GALACTOSIDASE

(57) Abstract: The present invention relates to a process for the production of a novel oligosaccharide, the process comprising combining a substrate with lactobacillus alpha-galactosidase enzyme. The invention also relates to the oligosaccharide itself and to compositions comprising it. The invention further relates to the use of the oligosaccharide, as well as compositions comprising it, for increasing beneficial bacteria in the gastrointestinal tract of an animal.

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FOOD PRODUCT AND PROCESS FOR THEIR PREPARATION BY ENZYMATIC GALACTOSYLTRANSFER WITH LACTOBACILLUS ALPHA-GALACTOSIDASE

The present invention relates to a process for the production of a novel oligosaccharide, the process comprising combining a substrate with an α-galactosidase enzyme. The invention also relates to the oligosaccharide itself and to compositions comprising it. The invention further relates to the use of the oligosaccharide, as well as compositions comprising it, for increasing beneficial bacteria in the gastrointestinal tract of an animal.

Until recently, oligosaccharides have been used in the food industry as a source of energy or as sweeteners. Nowadays, knowledge of their biological function and their role in cell-surface interactions has opened a new field of glycotechnology. Apart from their traditional use, oligosaccharides find new applications as immunostimulating agents or prebiotic compounds able to modulate the colonic microflora towards a healthy balance. This usually involves aiming to selectively increase the levels of bifidobacteria and lactobacilli at the expense of less desirable bacteria.

α-galactooligosaccharides as additives in functional food are of great interest because their α-galactosidic linkages are non-digestible by humans and monogastric animals and therefore reach the colon intact, where few bacteria are known to exhibit high α-galactosidase activity. However, access to a wide variety of original oligosaccharide or glycoconjugate structures is very limited. Extraction from their natural sources produces low concentrations of the oligosaccharide in the end product, while the chemical synthesis of oligosaccharides is a very complicated and labour intensive multi-step process.

The use of enzymes in synthesis of complex carbohydrates offers several advantages over chemical methods. A wide variety of regiospecific and often regioselective

reactions can be catalysed very efficiently without protection of the hydroxyl groups; these take place under mild conditions, often at room temperature and close to neutral pH, and organic solvents and hazardous chemicals or catalysts can be avoided. In nature, oligosaccharides are synthesised by glycosyltransferases. Glycosyltransferases catalyse the transfer of a glycosyl group from a glycosyl donor to an acceptor. They form regio- and stereospecific linkages between the activated donor and acceptor.

In looking at prebiotic compounds, the aim is to increase the level of beneficial bacteria in the gastrointestinal tract (GI). Lactic acid bacteria are food-grade microorganisms, generally regarded as safe, which can contribute towards the taste, smell or preservation of food products. *Lactobacillus* species have been found in large numbers as part of the intestinal flora of humans and other animals, where they are thought to increase resistance to common intestinal disorders, especially those with a microbial pathogenesis e.g. gastroenteritis. They can achieve this by fortifying the normal microflora either through their fermentation products or by the production of glycosidases, which degrade carbohydrates, thereby supplying energy for the growth of other bacteria. One *Lactobacillus* of particular interest according to the prevent invention is *Lactobacillus reuteri*. *Lactobacillus reuteri*, until recently misclassified as *Lactobacillus fermentum*, is hetero-fermentative and the only *Lactobacillus* species thought to inhabit the gastrointestinal tract of all vertebrates and mammals. *L. reuteri* has been reported to be one of the few lactobacilli isolated from the small and large intestinal mucosae and has been suggested that it produces cell surface proteins with mucus-binding properties. It also produces anti-microbial substances such as reuterin and reutericin, which are active against a range of Gram positive and Gram negative pathogenic bacteria.

This invention relates to production and properties of the α-galactosidase from *Lactobacillus* to be used for synthesis of novel oligosaccharide structures. Given that synthesis reactions with glycosyltransferases are kinetically controlled and that enzymes from different micro-organisms display differing kinetics for specific

glycosidic linkages, the synthesis products are able to confer selectivity at species level enhancing the specificity of prebiotics.

It is generally accepted that the bacterial community resident in the GI tract has a major impact on gastrointestinal function and thereby on the host's health and well being. The mammalian GI tract is a complex microbial ecosystem with the majority present in the large intestine, making the colon the body's most metabolically active organ. Because of the diversity and metabolic capabilities of the microflora, gut fermentation is a complicated process where the metabolic end products exerted by one individual species serve as a growth substrate for another.

Growth substrates for colonic bacteria include proteins, amino acids, bacterial secretions, lysis products and mucins, but the principal substrates are carbohydrates. These carbohydrates include mainly starches and dietary fibre, as well as oligosaccharides that have escaped digestion in the upper GI trade. These materials are firstly degraded by bacterial glycosidases, proteases and amino-peptidases to smaller oligomers and their sugar and amino acid components. These intermediates are then fermented by colonic bacteria to short chain fatty acids (SCFA), organic acids, gases and other end products.

In general, bacterial fermentation in the gut, can have either health promoting effects (production of butyrate) or harmful ones, and the interest is to modulate the gut microflora in such a way that the health promoting effects become predominant. This could be achieved through the use of probiotics and/or prebiotics.

The potential of exploiting the synergy between prebiotic and probiotic ingredients for increased potency of functional foods is finding its way in this area. This synbiotic concept involves a useful probiotic, incorporated into an appropriate dietary vehicle, and a suitable prebiotic. It is based on the fact that both the structure of the carbohydrate and the bacterial species present in the ecosystem are important factors

for the management of gut microflora, and aims to enhance the probiotic survival in the hostile environment of the colon by offering an available selective substrate. A synbiotic can be defined as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplement in the GI tract" (Gibson and Roberfroid, 1995, Journal of Nutrition, 125, 1401-1412).

The present invention uses glycotechnology in order to design and synthesise synbiotics consisting of a probiotic and a prebiotic tailored towards the specific organism. This approach takes into consideration the glycosidase specificity of the probiotic microorganism, of which the level of expression and pattern of linkage will probably be unique. This glycosidase profile can be used to design an oligosaccharide mixture, tailored to that specific probiotic, which will contain monosaccharides in linkages susceptible to faster hydrolysis by the glycosidases of this microorganism. The present invention also describes using enzymes produced by that probiotic to synthesise prebiotics, which in principle would act as a highly selective substrate.

The present invention therefore also provides utilisation of α-galactooligosaccharides produced via the transferase activity of an α-galactosidase produced by a *Lactobacillus*.

Accordingly, the present invention provides a process for the production of an oligosaccharide, the process combining an α-galactose substrate with an *Lactobacillus* α-galactosidase, under conditions which allow glycosyl transfer by the α-galactosidase and obtaining one or more oligosaccharides.

The α-galactosesubstrate may be any suitable substrate, and, in particular, may be one or more of raffinose, stachyose or melibiose.

The *Lactobacillus* α-galactosidase may be provided in a cell free extract or may be provided by the presence of *Lactobacillus* cells.

The oligosaccharide produced may comprise one or more sugar units, including one or more of glucose, galactose, fructose or a disaccharide.

The *Lactobacillus* α-galactosidase may be from any species of *Lactobacillus*, including *acidophilus*, *mucosae*, *reuteri*, *ruminus*, or *murinus*.

In particular, the oligosaccharide produced may comprise one or more of the following fractions:-

α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*; or
α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*

Such fractions are novel oligosaccharides.

A second aspect of the present aspect provides a novel oligosaccharide, obtainable by a process according to the first aspect of the invention.

A third aspect of the invention provides an oligosaccharide which comprises one or more of the following fractions:-

α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*; or
α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*

A fourth aspect of the invention provides a composition which comprises an oligosaccharide according to the third or fourth aspects of the invention. Such a composition may include one or more other oligosaccharides or sugars. The

composition may be in the form of a foodstuff.

A fifth aspect of the invention provides that oligosaccharide, according to the second, third or fourth aspect of the invention, for increasing beneficial bacteria in the gastrointestinal tract of an animal.

A sixth aspect of the invention provides a synbiotic which comprises an oligosaccharide according to the second or third aspects of the invention and a probiotic microorganism. The probiotic microorganism may be a *Lactobacillus*. The *Lactobacillus* probiotic microorganism may be any species, such as *reuteri*, *acidophilus*, *ruminus*, *murinus* or *mucosae*.

The synbiotic may be in the form of a foodstuff.

According to the present invention, the foodstuffs may be in the form of a drink, a dry, wet or semi-moist foodstuff.

The second, third, fourth and sixth aspects of the invention may form part of a pet food composition. Such a pet food of the present invention is for a pet animal. The pet animal of the present invention is preferably a mammal, most preferably a mammal having a single stomach as seen in a dog or a cat. The pet animal is preferably a cat or a dog. Cats and dogs according to the present invention are preferably *Felis silvestris catus* or *Canis familiaris*.

The form or type of the pet food composition is not limiting. It may be packaged. In this way, the consumer is able to identify, from the packaging, the ingredients in the food product and confirm that it is suitable for the particular pet animal in question. The packaging may be metal (usually in the form of a tin or flexifoil), but preferably plastic, paper or card. The pet food product may be a dry, semi-moist or a moist (wet) product. Wet food includes food which is sold in tins and has a moisture content from

about 70 to 90%. Dry food includes food having a similar composition, but with from about 5 to 15% moisture and presented as small biscuit-like kibbles. Semi-moist food includes food with a moisture content of between around 15% to 70% moisture. The amount of moisture in any product may influence the type of packaging which can be used or is required.

In combination with the probiotic strain (if the food is a synbiotic), the remaining components of the pet food composition are not essential to the invention and typical standard products can be combined with the required non-digestible carbohydrate. Most preferably, the combined ingredients of the pet food product according to the invention provide all of the recommended vitamins and minerals for the particular pet in question (a complete and balanced food), for example, as described in National Research Council, 1985, Nutritional Requirements for Dogs, National Academy Press, Washington D.C. (ISBN: 0-309-03496-5); National Research Council, 1986, Nutritional Requirements of Cats, National Academy Press, Washington D.C. (ISBN: 0-309-03682-8) or Association of American Feed Control Officials, Official Publication 1996.

The pet food composition (product) according to the present invention encompasses any product which a pet consumes in its diet. Thus, the invention covers standard food products as well as pet food snacks (for example, snack bars, pet chew, crunchy treat, cereal bars, snacks, biscuits and sweet products). The food product is preferably a cooked product. It may be in the form of a gelatinized starch matrix. It may be in the form of chunks in gravy, jelly, loaf or water. It may incorporate meat or animal derived material (such as beef, chicken, turkey, lamb, pork, fish, blood plasma, marrow bone etc or one or more thereof). The product alternatively may be meat free (preferably including a meat substitute such as soya, maize gluten or a soya product in order to provide a protein source). The product may contain additional protein sources such as soya protein concentrate, milk proteins, gluten etc. The product may also contain a starch source such as one or more grains (e.g. wheat, corn, rice, oats, barley

etc), or may be starch free. A typical dry or semi-moist dog or cat food may contain about 20-30% crude protein and about 10-20% fat, the remainder being carbohydrate, including dietary fibre and ash. A typical wet, moist or semi-moist product may contain (on a dry matter basis) about 40% fat, 50% protein and the remainder being fibre and ash.

The synbiotic may have the probiotic strain distributed throughout (such as mixed in to a canned or other product, optionally after opening) or may have the probiotic strain limited to a particular portion. For example, the probiotic strain may be a coating, such as sprayed onto the outside of a biscuit-like kibble. Alternatively, the probiotic may form part of an inner centre of a filled food product, such as described in WO 99/47000 or in EP-A-0,647,410.

The pet food composition is preferably a commercial pet food product. Such a product is preferably sold as a product for feeding to a pet animal, in particular a pet cat or a pet dog.

The pet food composition may be in the form of a drink. Where such a composition is a synbiotic composition, the probiotic strain is mixed with the other drink ingredients, such as milk or water. The composition may also be in the form of a food supplement. The food supplement is preferably in a form which can be added to a food product for consumption by the animal. The food supplement may be a lipid/fat/oil or water-based formulation (for example as a liquid or semi-liquid format). It may be encapsulated, or in the form of a tablet, or in the form of a dried and/or freeze-dried powder. Such a powder or the liquid or semi-liquid supplement may be encapsulated in an edible coating or in, for example, a paper or plastic sachet. Preferably, the pet food composition provides nutritional ingredients to the pet animal in addition to the probiotic microorganism, such as in the form of a pet treat or a complete and balanced pet food (dry, wet or semi-moist). This avoids the need to further supplement the pet's diet.

The pet food composition of the present invention may comprise one or more probiotic strains. Such strains are preferably selected from *Lactobacillus, Lactococcus, Micrococcus, Bifidobacteria* or *Enterococcus*.

In the foodstuff of the present invention the *Lactobacillus* strain is preferably present at a concentration of from 10^3 to 10^{15} viable cells per gram of the total composition. This concentration of cells provides a suitable concentration for successful colonisation of the gastrointestinal tract and providing the desired health benefits to the animal. An additional probiotic strain may be present at a concentration of from 10^3 to 10^{15} viable cells per gram of the total composition.

An advantage of the present invention is that the pet food composition comprises a prebiotic, in addition to the probiotic. Accordingly, if a prebiotic is provided alongside the probiotic in a pet food composition, the probiotic is thus provided with a preferred metabolite. A mixture of a prebiotic and a probiotic strain that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract is often referred to as a synbiotic. Any synbiotic according to the present invention may additionally comprise one or more of chicory fibre, coconut endosperm fibre, rice bran, beet fibre (such as sugar beet pulp), carob bean or gum talha. Also included are saccharides, including oligosaccharides.

Examples of suitable oligosaccharides include one or more of: Fructooligosaccharide, Biotose (malto syrup), Palatinose, IMO (Iso Malto Oligosaccharides), Cellobiose, Gentibiose, Inulin, Laevan, Maltodextrin, Maltose, Melibiose, Raffinose, Lactose, Panorich (panose-rich syrup), Melezitose, Stachyose, Sucrose or Xylan. The saccharides may be in purified or non-purified form (such as raw plant material).

The combination of the probiotic strain and the prebiotic ingredient increases the effectiveness of anti-pathogenic activity of the probiotic strain.

The foodstuffs of the present invention provide a health benefit to animals. The health benefit may be one or more of the following nutritional and therapeutic benefits:-

Microbial ecology of the intestinal flora

- Promotion and maintenance of host health
- Stabilisation of a balanced gut microflora

Antimicrobial effects

- Lactic, volatile acid production
- Hydrogen peroxide, CO_2, diacetyl production
- Bacteriocin production

Pathogenic interference

- Competitive exclusion of enteric pathogens and reduction in diarrhoea

Physiological effects

- Improvement of nutritional quality of food and feed
- Extended survival time of the host
- Metabolic stimuli of vitamin synthesis and enzyme production
- Reduction in lactose intolerance
- Reduction of serum cholesterol by assimilation mechanisms

Immunomodulatory

- Enhanced innate host defences
- Stimulation of phagocytosis by peripheral blood lymphocytes
- Modulation of cytokine gene expression

Anti-tumour effects

- Detoxification/binding of carcinogens
- Anti-mutagenic activity
- Tumour suppression by modulation of cell-mediated immunity

Management of diarrhoea

- Antibiotic-induced

A seventh aspect of the present invention provides a process for the preparation of a composition or synbiotic according to any one of the aspects four or six.

The process comprises combining the oligosaccharide with the other components and optionally heating and/or forming the combination into a product. The combination of the probiotic strain with the pet food ingredients may be in a mixture or may be in a more precise format. For example, the probiotic strain may be applied to the outside of the formed composition or may be part of an internal filling. The optional heating and/or forming the composition may apply to a combination of the probiotic strain and the other components. Alternatively, the other components may be optionally heated and/or formed before application of the probiotic strain. Standard techniques and procedures as known in the art may be used, such as those described in US 5,968,569, ZA 975040, US 3,916,029, US 4,900,572, EP-A-0,258,037, US 4,997,671, US 6,117,477 and WO 98/05219, the content of each of which is incorporated herein by reference. The other components comprise other ingredients when the composition or synbiotic is a foodstuff.

The process may also include packaging the product. It may include canning or adding the ingredients to flexifoils or bags (paper or otherwise). The process may include extruding the product. The process may include drying the product.

An eighth aspect of the present invention provides a method of obtaining a health benefit in an animal, the method comprising administering an oligosaccharide, composition or synbiotic as per the second, third, fourth or sixth.

The method is particularly useful where the animal is in need of a health benefit. The health benefit may be prophylactic or therapeutic.

All preferred features of individual aspects apply to other aspects, *mutatis mutandis*.

The invention is described with reference to the drawings, in which:

Figure 1 is a time course of melibiose conversion and oligosaccharide synthesis starting from 23% (w/v) melibiose concentration (▲, melibiose; ♦, galactose and glucose; ■, α-D-Gal*p*-(1→6)-α-D-Gal*p*; •, α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*; ✳, α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*)

Figure 2 is a time course of raffinose conversion and oligosaccharide synthesis starting from 23% (w/v) raffinose concentration (◇: Fructose, x: Galactose and Glucose, + α-D-Gal*p*-(1→6)-α-D-Glc*p*, : Sucrose, ■: α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*,△: Raffinose, ✳: Stachyose and ●: Pentasaccharise)

Figure 3 is synthesis of oligosaccharides by *L. reuteri* α-Galactosidase as a function of substrate concentration (Mellibioe ♦ ; Raffinose ■).

Figure 4 shows the differences in the bacterial population size (▒ FOS; ▯ GMM; ▒ GMMa; ▉ GMMr) compared to the total bacteria counted ({Selected bacterial numbers at 24h/ total bacteria counted at 24h}- ({Selected bacterial

numbers at 0h/ total bacteria counted at 0h}) and differences in the bacterial population numbers (Δ FOS; o GMM; × GMMa; □ GMMr) of the monitored genera after 24h of fermentation.
(M, mellibiose; R, raffinose; FOS, Fructooligosaccharides; GMMa, GMM + *L. acidophilus*; GMMr, GMM + *L. reuteri*)

Figure 5 shows the production of lactic acid, acetic acid, propionic acid and butyric acid by canine gut microflora at 5 (▒), 10 (■) and 24 h (▓) in stirred pH controlled batch cultures. Values are means of triplicates with standard deviation represented by vertical bars.
M, mellibiose; R, raffinose; FOS, Fructooligosaccharides; GMMa, GMM + *L. acidophilus*; GMMr, GMM + *L. reuteri*.

The present invention will now be described with reference to the following non-limited examples:

Examples

Example 1

Synthesis of α-galactooligosaccharides with α-galactosidase from *Lactobacillus reuteri*.

Materials and Methods

Organism and medium

Lactobacillus reuteri was obtained from the culture collection of the Waltham Centre for Pet Nutrition (Waltham-on-the-Wolds, Leicestershire, UK). The working culture was maintained in MRS broth (Oxoid, Basingstoke, Hampshire, UK) and MRS agar (MRS broth plus 1.5%, w/v, agar) stabs. The pH of the growth medium was adjusted

to 6.7 with 0.1 N NaOH before autoclaving (121°C for 15 mins).

Effect of carbohydrates on α-galactosidase production

An overnight culture of *L. reuteri* grown in MRS broth at 37°C under anaerobic conditions (10:10:80; H_2:CO_2:N_2) was harvested by centrifugation (30000 x g for 20 min). After two washes with sterile 0.02M phosphate buffer (pH 6.8), the cell pellet was resuspended in the same buffer and used to inoculate (1% v/v) glucose free-MRS broth containing one of the following carbohydrates as sole carbon source: maltose, cellobiose, lactose or raffinose (final concentration 2%, w/v). Production of α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase in these cultures was then assayed. Bacterial growth was determined by absorbance measurement (A_{550}) with conversion to colony forming units (c.f.u.) by means of a calibration curve.

Preparation of crude extract and α-galactosidase assay

L. reuteri glycosidases (data not shown) were located in the cell debris fraction of the crude extract. At various intervals during cultivation, 20 ml of culture was sampled and centrifuged at 30,000 x g for 20 min and the cell pellet washed twice with 0.2M potassium phosphate buffer (pH 6.8). The cell pellets were resuspended in the same buffer and intracellular enzyme was released by the addition of 10,000 units/ml of lysozyme with incubation at 37°C for 2h. After centrifugation (30,000 x g for 30 min), the supernatant was assayed for intracellular α-glucosidase, α-galactosidase and β-galactosidase activities. Glycosidase activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl-p-α-D-glucopyranoside, *p*-nitrophenyl-p-β-D-glucopyranoside, *p*-nitrophenyl-p-α-D-galactopyranoside and *p*-nitrophenyl-p-β-D-galactopyranoside. 100µl of crude extract was mixed with 300µl of the substrate solution (5mM) in 0.2M potassium phosphate buffer (pH 6.8) and the mixture incubated at 37°C for 2 min. A solution of 2ml disodium tetraborate (0.2M) was then added to the mixture to stop the reaction. The *p*-nitrophenol released was read at 400nm in a spectrophotometer. Specific enzyme activity was defined as 1µmol

p-nitrophenol liberated from the substrate per mg of protein per min.

α-galactosidase activity against melibiose and raffinose was measured by HPLC as a decrease in the relative area of the oligosaccharide peak for the formation of galactose. The assays were carried out three times.

Determination of the optimal pH and temperature for α-galactosidase activity

The optimum pH and temperature for enzyme activity were determined by adding 100µl of enzyme extract in 0.2M phosphate buffer (pH 6.8) to 300µl of 5mM *p*-nitrophenyl-α-D-galactopyranoside, 5mM melibiose or 5mM raffinose in one of the following buffers: 0.2M citrate phosphate (pH 4.5-6.8) and 0.2M phosphate (pH 6.8-8). Incubation was carried out at temperatures between 25 and 60°C. In the case of *p*-nitrophenyl-α-D-galactopyranoside the reactions were stopped by the addition of 2ml of 0.2M disodium tetraborate and enzyme activity was then assayed spectophotometrically at 400nm. When melibiose and raffinose were used as substrates the reactions were stopped by boiling the sample for 3 min and the enzyme activity was assayed by HPLC. Protein content of the extract was determined using the bicinchoninic acid assay with BSA as a standard. The optimum temperature and pH for the transferase activity of the α-galactosidase was determined by incubation of 300µl melibiose (5mM) or raffinose (5mM) with 300µl of cell-free extract in buffer pH 6.0-7.8 at 25°C, 35°C and 45°C for 3h.

Purification of α-galactosidase from *L. reuteri*

Purification of α-galactosidase from crude enzyme extracts involved ultrafiltration through a 100kDa nominal molecular weight cut-off (NMCO) MX 50 (Osmonics Desal, Le Mee sur Seine, France) membrane in a Gyrosep™300 stirred cell (Techmate Ltd, Milton Keynes, UK) and size-exclusion chromatography on a column (900mm x 250mm) of Sephacryl S-300. The protein was eluted with 0.1M Tris HCl buffer (pH 8.0) and the fractions collected were assayed for α-galactosidase activity.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed in 12% (w/v) polycrylamide gels containing 0.1% (w/v) SDS according to the method of Laemmli. The gels were strained with Coomassie brilliant blue for 30 min.

Transgalactosylase activity of the cell free extracts

A mixture of 3ml of substrate at 11.5 to 68.6% (w/v) final concentration in potassium phosphate buffer (0.2M) containing 0.001% (w/v) sodium azide was incubated with 2.7ml of crude extract. When raffinose was serving as a substrate, the reaction was carried out at pH 7.8 and 45°C, while for mellibiose the reaction was carried out at pH 7.2 and 35°C. At intervals, a portion of the reaction mixture was removed and heated in boiling water for 8min. The samples were analysed by HPLC.

HPLC

HPLC was performed using a Merck-Hitachi LaChrom system (Merck, Poole, Dorset, UK) equipped with an APEX Carbohydrate column (Jones Chromatography, Mid Glamorgan, UK) and a Merck-Hitachi LaChrom RI detector. 70% (v/v) acetonitrile was used as the eluent at 25°C and a flow rate 0.8 ml/min.

Methylation analysis

Linkage positions for the respective galacto-oligosaccharides preparations were determined by methylation analysis. The freeze-dried samples (5-6mg) were dispersed in dry dimethyl-sulfoxide (DMSO) at 20°C for 16 h after flushing with argon. They were methylated by sequential addition of powdered sodium hydroxide (0.5g) and iodomethane (4ml) (7,18). After elution-extraction on a C18-bonded cartridge (Sep-Pak, Waters, Watford, UK), the methylated carbohydrates were dried, extracted into $CHCl_3/CH_3OH$ (1:1, v:v), and evaporated to dryness. The samples were hydrolysed using trifluoroacetic acid, and converted to partially methylated alditol acetates (PMAAs) by $NaBD_4$ reduction and acetylation with acetic anhydride and

N-methylimidazole. The PMAAs were analysed by GC on a cross-bonded 50% cyanopropyl methyl-50% phenyl methyl polysiloxane column (Thames Chromatography, Maidenhead, UK) using a flame ionisation detector and a temperature program: 55°C (2 min), +45°C min^{-1} (1.9 min), 140°C (2 min), +2°C min^{-1} (35 min), 210°C (40 min). The PMAAs were identified by measuring their retention times relative to *myo*-inositol hexaacetate, and comparing the relative retention times with those of external standards. A mixture of standards for each sugar was prepared by deliberate under-methylation of methyl glycosides. Peak areas were represented as relative molar quantities using effective carbon response factors.

Identities of PMAAs were confirmed by their electron-ionisation mass spectra. GC-MS analysis was performed on an identical GC in series with a Fisons Analytical Trio 1S mass spectrometer, using a source temperature of 200°C and an ionisation potential of 70eV.

Results

Effect of carbohydrates on growth and enzyme production by *L. reuteri*

L. reuteri assimilated maltose, lactose and raffinose well, but only showed poor assimilation of cellobiose. With all three carbon sources that supported growth, the principal enzyme produced was β-galactosidase.

α-galactosidase was present at high levels in the enzyme extract produced with raffinose as the carbon source, showing a 7-fold and 14-fold increase in enzyme activity when compared to extracts from bacteria grown on lactose and maltose respectively.

α-Galactosidase expression began at the start of exponential phase, reaching a maximum by stationary phase, after which a rapid decrease in activity was seen.

Characterisation of α-galactosidase from *L. reuteri*

The cell free extracts exhibited activity in a pH range between 4.5 and 6.8. The optimum pH was between 4.5 and 5.0, in agreement with past reports which found that pH values below 6.0 were optimal for α-galactosidase activity in hetero-fermentative lactobacilli.

The effect of temperature on α-galactosidase activity in hetero-fermentative lactobacilli.

The effect of temperature on α-galactosidase activity of the microorganism was determined. Optimum temperature was found to be 50°C, while incubation at 60°C for 10 min gave a complete inactivation of the enzyme. Similar results have been reported by other authors.

Typical Michaelis-Menten kinetics were observed when the rate of substrate hydrolysis was plotted against substrate concentrations. The K_m was 0.55mM and the V_{max} was 0.80 μmol/min per mg of protein at pH 5.0 and 50°C, as determined by Lineweaver-Burk plot. The enzyme was purified to homogeneity from the cell free extract of the microorganism grown on raffinose (Table 1). The final protein gave a single band on SDS-PAGE of an estimated molecular mass of about 64kDa.

Transgalactosidase activity of cell free extract

Transferase activity of the cell free extract was studied in the presence of mellibiose or raffinose (Table 2). Activity was confirmed by the appearance of synthesis products in the presence of melibiose and raffinose.

The optimal conditions for transferase activity with melibiose as the substrate, were at pH 7.2 at 35°C, while with raffinose as substrate optimal conditions were pH 7.8 at

45°C (Table 2). The ratio of the transferase activity over the hydrolytic activity for both substrates was the highest at pH 7.8 and 35°C, although synthesis yields at these conditions compared to the optimal conditions were 85% for melibiose and 42% for raffinose.

The conversion of melibiose and raffinose to hydrolysis products and synthesised oligosaccharides with time in a typical experiment, starting from 23% (w/v) melibiose or 23% (w/v) raffinose concentration, is presented in Figs 1 and 2. Melibiose was hydrolysed to glucose and galactose and, in addition oligosaccharides were produced reaching a yield of 23% (w/v). The synthesised oligosaccharides, based on the retention times of the HPLC, could be distinguished in a disaccharide and two different trisaccharide fractions (Fig 1). α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p* (Table 3). The presence of fructose in the hydrolysis products is explained by the presence of an active fructofuranosidase enzyme in the crude enzyme extract of *L. reuteri*.

In both cases, synthesis ceased when hydrolysis of the substrate reached approximately 50%.

When different substrate concentrations were used, the effect was mainly on the time that the maximum yield was observed (increased incubation time was needed for higher substrate concentrations). For both substrates the synthesis yield was almost the same for substrate concentrations between 20-70% (w/v) (Fig 3). The maximum synthesis yields were seen at 23% (w/v) melibiose, yielding almost 26% (w/w) for the trisaccharide fraction after 8h of incubation (Fig 1). In the case of raffinose, maximum synthesis yields were achieved at 23% (w/v) substrate after 7h, yielding 18% (w/w) of synthesis product (Fig 2).

Discussion

α-galactooligosaccharides, particularly raffinose and stachyose, have been described as prebiotic substrates promoting the growth of probiotic bacteria in the colon while other intestinal bacteria, such as *Bacteroides* spp. and *Clostridium* spp. do not assimilate these oligosaccharides as effectively.

Since α-galactosides seem to be very promising substrates for *L. reuteri*, we focused on the production of new α-galactosides using the α-galactosidase from this microorganism.

To our knowledge, no α-galactosidase isolated from lactobacilli has been used in synthesis experiments, although these bacteria are able to ferment α-galactosides. *L. reuteri* α-galactosidase was found to synthetically act at the C-6 hydroxyl group of the galactosyl residue in both raffinose and melibiose transgalactosylation reactions without any other linkages formed. In the presence of raffinose the main synthesised product was stachyose; in the presence of melibiose the synthesised products were of α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p* and α-D-Gal*p*-(1→6)-α-D-Gal*p*.

Table 1. Purification of α-galactosidase from *Lactobacillus reuteri*.

Step	Specific Activity (units/mg of Protein)	Total Activity (units)
Cell extract	0.27	590
Ultrafiltration	0.96	427
Size exclusion Chromatography	3.42	182

Table 2. Specific hydrolytic and transferase α-galactosidase activity at various pH and temperatures with melibiose and raffinose as substrate

		Specific α-galactosidase activity (μmol of substrate used mg^{-1} of protein h^{-1})					
		Hydrolytic activity			Transferase activity		
Substrate	**pH**	25°C	35°C	45°C	25°C	35°C	45°C
Melibiose	5.8	2.31	3.21	2.44	1.17	3.32	2.97
	6.4	6.74	7.13	4.12	1.34	4.24	2.63
	7.2	1.98	5.10	1.00	1.88	4.76	0.83
	7.8	1.33	2.94	0.85	1.39	4.11	1.54
Raffinose	5.8	1.15	2.82	9.65	0.65	3.13	7.38
	6.4	1.22	3.42	10.80	0.74	3.63	10.43
	7.2	0.92	3.25	9.24	1.46	4.87	14.33
	7.8	0.58	2.19	7.22	1.93	6.92	16.13

Table 3. Linkage molar ratios for synthetic oligosaccharide mixtures determined by methylation analysis

	Molar ratio for mixture[α]		
Residue and Linkage	1	2	3
t-Man*p*	ND	ND	0.56
t-Glc*p*	ND	ND	0.55
t-Gal*p*	1.00	1.00	1.00
1,6-Glc*p*	0.63	1.05	1.1
1,6-Gal*p*	1.72	0.41	0.90

[α]Oligosaccharide mixtures were as follows: 1, synthesis product, when meliiose was

used as the reaction substrate, containing two disaccharides at 63:37 ratio; 2, synthesis product, when raffinose was used as the reaction substrate containing melibiose and one trisaccharide at 85:15 ratio; 3, synthesis product, when raffinose was used as the reaction substrate containing a tetrasaccharide; ND, not detected. The degree of polymerisation for its oligosaccharide was determined by HPLC.

Example 2

In vitro evaluation of the fermentation properties of galactooligosaccharides synthesised by α-galactosidase from *L. reuteri*.

Abstract

Stirred, pH controlled anaerobic batch cultures were used to evaluate the *in vitro* utilisation by canine gut microflora of novel α-galactooligosaccharides synthesised with an enzyme extract from a canine *Lactobacillus reuteri* strain. Fructooligosaccharides (FOS), melibiose and raffinose were used as reference carbohydrates for the prebiotic properties of the synthesised oligosaccharide (GMM). Addition of *Lactobacillus acidophilus* was used as control for the evaluation of the synbiotic properties of the oligosaccharide with *L. reuteri*. Populations of predominant gut bacterial groups were monitored over 48h of batch culture through fluorescent *in situ* hybridisation and short-chain fatty acid (SCFA) production was measured. GMM showed higher increase in bifidobacteria and lactobacilli population number and size as well as a higher decrease in clostridia population number and size compared to the commercial prebiotics (FOS, melibiose, raffinose). That prebiotic effect was further increased by the addition of *L. reuteri* followed by a change in the SCFA production pattern compared to just GMM and GMM with *L. acidophilus*. The observed change in the SCFA production was in accordance with the fermentation properties of *L. reuteri* suggesting that the novel synbiotic had a significant effect in the canine gut microflora fermentation.

Materials and Methods

Chemicals. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Co. Ltd. (Poole-UK) and bacteriological growth media supplements were obtained from Oxoid Ltd. (Basingstoke, UK).

Substrates and bacterial strains. *Lactobacillus acidophilus* and *Lactobacillus reuteri* were obtained from the culture collection of the Waltham Centre for Pet Nutrition (Waltham-on-the Wolds, Leicestershire, UK).

Mellibiose, raffinose and Raftilose P95 (Fructooligosaccharides (FOS), Orafti, Tienen, Belgium) were commercial oligosaccharide preparations. 6-Galactosyl mellibiose mixture (GMM) was prepared by the transgalactosidase activity of an α-galactosidase produced by *L. reuteri*. The compositions of these products are shown in Table 4.

Preparation and collection of fecal samples. Fresh fecal samples were obtained from one healthy dog maintained on a complete daily diet. The samples were collected at WCPN and maintained under anaerobic conditions. A 1/10 dilution in anaerobic phosphate buffer (0.1M, pH 7.4) was prepared and the samples homogenized in a stomacher for 2 minutes.

Batch fermentations. Sterile, stirred, batch culture fermentation vessels (300ml volume) were filled with 135ml basal nutrient medium (peptone water 2g/l, yeast extract 2g/l, NaCl 0.1g/l, K_2HPO_4 0.04g/l, KH_2PO_4 0.04g/l, $MgSO_4.7H_2O$ 0.01g/l, $CaCl_2.6H_2O$ 0.01, $NaHCO_3$ 2g/l, Tween 80 2ml, Hemin 0.02g/l, Vitamin K_1 10µl, Cysteine.HCl 0.5g/l, Bile salts 0.5g/l, pH7.0), autoclaved at 121°C for 15 minutes and gassed overnight with O_2-free N_2 (15 ml/min). Before addition of the fecal slurry, the oligosaccharide mixtures were added through a 0.2µm filter to give a final concentration of 1% w/v, the temperature was set at 37°C by means of a circulating water bath and culture pH was maintained at 6.8 in all vessels using a pH controller.

The vessels were inoculated with 15ml of fresh fecal slurry (10% w/v) and continuously sparged with O_2-free N_2 at a rate of 15 ml/min. For the vessels that received the test synbiotics , lactobacilli cells (10^9 cfu/ml) were added to the fecal slurry at a final inoculum concentration of 1% v/v. Five ml samples from each vessel were obtained for fluorescence *in situ* hybridization (FISH) and analysis of SCFA by high performance liquid chromatography (HPLC). The batch culture fermenters were run over a period of 48 hours and samples were obtained at the start (T0), after 5h, 10h, 24h and at the end of the incubations (48h).

Bacterial enumeration. Differences in bacterial populations were assessed using FISH with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesized and labeled with the fluorescent dye Cy3 (Eurogentec UK Ltd). The probes used were Bif164, Bac303, Lab158, His150, Ec1531 and *Eubacterium*, specific for bifidobacteria, bacteroides, *Lactobacillus/Enterococcus* spp., clostridia (*Clostridium perfringens /histolyticum* subgroup), *E. coli* and *Eubacterium* spp. respectively. For total bacterial counts the nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI) was used. Samples obtained from fermentation vessels were diluted four times in 4% (w/v) paraformaldehyde and fixed overnight at 4°C. The cells were then centrifuged at 1500 x g for 5 minutes, washed twice with phosphate-buffered saline (PBS; 0.1M, pH 7.0), re-suspended in a mixture of PBS and 99% ethanol (1:1 v/v) and stored at –20°C for at least 1 hour. The cell suspension was then added to the hybridization mixture and left to hybridize at the appropriate temperature for each probe (10, 12, 8, 2, 16). Hybridized mixture was vacuum filtered using a 0.2µm Isopore membrane filter (Millipore Corporation, Herts, UK). The filter was removed, placed onto a glass slide with SlowFade (Molecular Probes, Eugene, OR, USA) and examined under a fluorescent microscope (Nikon Eclipse, E400). The DAPI stained cells were examined under UV light and hybridized cells viewed using a DM510 filter. For each slide sample at least 15 different fields of view were counted.

Analysis of SCFA. The production of lactic, acetic, propionic and butyric acids in the fermentations was quantified. Samples were centrifuged at 1500 x g for 15 minutes and the resultant supernatant used for analysis. A Model 1050 UV high-pressure liquid chromatograph (Hewlett Packard) with an integrated oven compartment (50°C) and data system was used. Sample injection (20µl) was performed using an autosampler. The column was a pre-packed Aminex HPX-87-H strong cation-exchange resin column (150 x 7.8 mm I.D.), fitted with an ion exclusion micro-guard refill cartridge (Bio-Rad, Watford). The eluent was 0.005 M sulfuric acid.

Oligosaccharide mixture preparation. Purification of the oligosaccharide mixtures were carried out using a nanofiltration membrane (DS-5-DL) supplied by Osmonics Desal (Le Mee sur Seine, France). According to the manufacturer, the DS-5-DL membrane had a molecular weight cut off on sucrose and glucose and a 96% rejection of $MgSO_4$. The membrane was a three film composite membrane with a pH operation range between 1-11 and a maximum pressure and temperature of 27bar and 90°C respectively.

A High Pressure Cell Test Unit (Osmonics Desal; Le Mee sur Seine, France) consisting of a tri-piston pump, a feed tank and two stainless steel high pressure cross flow cells connected in parallel to the pump outlet was used for nanofiltration. Before separation of the feed stream from the pump to the two inlets of the NF cells, a pressure gauge was fitted to allow readings of the system pressure. The unit had a maximum operating pressure of 70bar, a maximum operating temperature of 90°C, a pH operating range of 1-13, and the pump had a feed flow rate of 220.8 L h^{-1}. The feed tank (2-5L capacity) had a vertically placed baffle, a cooling / heating coil connected to a temperature-controlled water-bath fitted to allow temperature control of the feed.

Before purification, membranes were conditioned by compressing them to a steady state of compaction (determined by the flux of permeate), with demineralised water as feed, at an intermediate pressure according to their pressure limits and at 25°C (7). For purification of the oligosaccharide mixtures, a 2L solution was added to the feed tank, the pressure set to 13.8bar and following total re-circulation mode of operation (permeates and retentates re-circulated to the feed tank) the temperature of the feed was increased to 60°C. This was in order to decrease the rejection given by the membrane for the monosaccharides in the solution and achieve improved purification. This effect of temperature on rejection of the sugar components of the mixture is more intense for lower molecular weight sugars. After the desired temperature was achieved the system was allowed to stabilize for 45min before a continuous diafiltration purification of the sugar mixture commenced. In continuous diafiltration, water at the appropriate pH and temperature is added to the feed tank at the same rate as the permeate flux, thus keeping the feed volume constant during processing (1). Equilibrium, between the water supplied (with a peristaltic pump) and the removed permeate in the continuous diafiltration purification, was achieved by adjusting the pump flow rate to the flow rate of the permeates as measured at different time intervals; by continuously controlling the amount of total cumulative permeate and water added, and by controlling feed volume in the feed tank. Throughout the purification process, 8L of permeate was removed and the purified oligosaccharide mixture consisted of 8% (w/v) monosaccharides, 20% (w/v) disaccharides and 72% (w/v) oligosaccharides.

Statistical analysis. Differences between bacterial counts at 0, 10 and 24h of fermentation for each batch culture run were tested for significance using paired *t*-test, assuming equal variances and considering both sides of the distribution (two-tailed distribution). Differences were considered significant at $P \leq 0.05$.

Results

Bacterial growth in pure cultures

L. acidophilus and *L. reuteri* were tested for their ability to ferment the synthesised oligosaccharide mixtures. Both lactobacilli were able to ferment the oligosaccharides with growth rates similar to those on mellibiose but lower than those on raffinose (Table 5).

Changes in bacterial population numbers

The recorded changes of selected bacterial populations in the mixed batch cultures with the oligosaccharides tested are shown in Tables 6 and 7. With all the oligosaccharides used, the total number of bacteria increased from the start of the fermentation reaching their highest numbers after 10h. The only addition that showed an increase in the population number of total bacteria for longer than the first 10h of fermentation was GMM+*L. reuteri* reaching the highest increase of 0.7 log after 24h. After 24h fermentation the bacteroides population numbers were similar to those at time 0, except when grown on raffinose for which numbers were seen to increase. Populations of bacteroides grown on the GMM+*L. reuteri* addition showed a significant increase for the first 10h followed by a similar decrease during the following 12h incubation. Clostridia were seen to decrease after 24h for the GMM and GMM+*L. reuteri* additions, but increased on raffinose and FOS. *E. coli* decreased in population numbers throughout the 24h of fermentation, except on the raffinose and GMM+*L. acidophilus* addition.

Eubacterium numbers showed an increase on raffinose and FOS, while for the synthetic oligosaccharides only those containing *L. reuteri* were seen to increase for the first 24h. The highest decrease in the *Eubacterium* population numbers was recorded for the synthetic oligosaccharide mixture (GMM).

Bifidobacterial counts showed a large significant increase in every case, with GMM+*L. acidophilus* and GMM+*L. reuteri* recording the highest increase of over 1 log after the first 24h.

Changes in the numbers of lactobacilli were, however, less pronounced with the GMM mixture, either alone or with the lactobacilli added, showing an increase of 0.25 log over the increase that could be observed when the commercial oligosaccharides were used.

The effect of various additions on the bacterial population size as a fraction (Fig. 4) of the total bacteria counted showed an increase in *Bifidobacterium* and *Lactobacillus* populations for all the additions, with the *L. reuteri* synbiotic having the most significant effect. Clostridia, *E. coli* and *Bacteroides* showed a reduction in their fraction of the total bacteria counted, while for eubacteria only raffinose and the *L. reuteri* synbiotic seemed to experience a positive effect. For all the bacterial groups monitored, effects on the population fractions were most profound in the presence of *L. reuteri*.

Changes in SCFA levels

Levels of SCFA measured in the fermentations are shown in Fig. 5. Lactate increased with all additions, except GMM and GMM+*L. acidophilus* for which no change could be seen after the first 5h of fermentation. In the presence of mellibiose and the addition containing *L. reuteri*, the increase could be observed for more than 10 h. Propionate concentrations showed an increase for the first 5h fermentation after which propionate levels remained stable for FOS and raffinose. The addition of GMM showed a significant decrease in propionate after the first 5 h, while for mellibiose the increase in propionate continued until 24h. Butyrate concentrations showed a decrease after the first 5h with all the additions, except mellibiose. Acetate concentrations remained stable for raffinose, GMM and the *L. acidophilus* addition, while a significant increase could be seen during the mellibiose fermentation. For FOS and the *L. reuteri* addition, acetate showed an increase up until 10 h incubation.

Discussion

Dietary composition is probably the most important controlling factor for microbial activity in the gastro-intestinal tract of non-ruminant animals. Undigested food that reaches the intestine determines the fate of the microbial population via the type of substrate that it provides. Despite a lack of understanding on ecological interactions between bacterial species in the colon of dogs, it is still possible to associate bifidobacteria and lactobacilli with a healthy hindgut. These bacterial genera may inhibit gut persistence by pathogens and are generally considered to have protective effects against acute and chronic gut disorders in humans. It is therefore desirable to increase populations of lactobacilli and bifidobacteria in the large intestine of dogs with probiotics, prebiotics and synbiotics. This study was designed to generate information on the fermentation properties of α-galactosyl oligosaccharides synthesised by α-galactosidases from *L. reuteri* and whether this mixture could be more effective when added in a synbiotic form containing the strain from which the synthetic enzymes originated.

From the data presented all four oligosaccharides displayed potentially prebiotic properties by increasing numbers of bifidobacteria and lactobacilli. Galactooligosaccharides as additives in functional foods are of interest because their α-galactosidic linkages are non-digestible by humans and monogastric animals and therefore reach the colon intact, where few bacteria are known to exhibit high α-galactosidase activity.

GMM showed a higher increase in the numbers of bifidobacteria and lactobacilli, than FOS, mellibiose and raffinose (Table 7), as well as in the population fractions of these genera compared to the total bacteria counted after 24h fermentation (Fig. 4). All of the oligosaccharides tested showed similar effects on bacteroides, while GMM, when compared to the commercial oligosaccharides, showed significant decreases in clostridia, *E. coli* and eubacteria (Table 7).

When *L. reuteri* was added to the fermenters, bifidobacteria and lactobacilli showed even higher increases than with the oligosaccharide mixture alone. This increase was higher for *L. reuteri* than for *L. acidophilus* of which the addition did not seem to have a synergistic effect with the oligosaccharide (Fig. 4).

Regarding the SCFA profile of these anaerobic fermentations, the addition of *L. reuteri* affected the production of lactate and acetate compared to both GGM preparations. Lactate in the presence of *L. reuteri*, showed a similar pattern of production as both GMM and GMM+*L. acidophilus* additions until 10h of fermentation, but after 24h a significant increase could be seen. A very significant increase in the production of acetate could be seen from the beginning of the fermentation until the first 10h, after which a slight decrease occurred. This pattern of acetate and lactate production can be attributed to the heterofermentative nature of *L. reuteri*, which has been reported to initially produce acetate followed by lactate as its principal fermentation end products. This suggests that the bacterial addition was present in the fermentation.

The present study has shown that the synthesized oligosaccharide mixture demonstrated prebiotic properties (i.e. increase in bifidobacteria and lactobacilli; decrease in clostridia and *E. coli*). The prebiotic effect was further magnified by the addition of *L. reuteri*, delivering the first canine synbiotic consisting of a prebiotic synthesized by enzymes extracted from the strain used as the probiotic.

Table 4. Structure and composition of oligosaccharides used in the present study

Substrate	Composition
Mellibiose	99% Mellibiose (Galα 1-6 Glc)
Raffinose	98% Raffinose (Galα 1-6Glcα 1-2βFru)
Raftilose P95	95% oligosaccharides (Glcα1-2[βFru1-2]$_n$ where n=2-9 average 4-5
GMM	8% Gal + Glc; 19% melibiose (Galα 1-6Glc); 72% oligosaccharides of which 85% Galα 1-6Galα 1-6Glc

Table 5. Growth rates of *Lactobacillus reuteri* and *L. acidophilus* in mellibiose, raffinose and the newly synthesized oligosaccharide mixture (GMM) followed spectophotometrically at A_{550}

Microorganism	Mean Growth Rate $\mu^* \pm$ SD		
	Mellibiose	Raffinose	GMM
L. reuteri	0.25±0.02	0.30±0.02	0.21±0.01
L. acidophilus	0.21±0.02	0.39±0.03	0.171±0.01

* Growth rate (μ) was calculated as log c.f.u. ml^{-1} h^{-1} (mean value of 5 replicate)

Table 6. Bacterial populations* in stirred, pH controlled batch culture fermentations

		M		R		FOS		GMM		GMMa		GMMr	
Group	Inoculum†	10	24	10	24	10	24	10	24	10	24	10	24
Total count	8.84	9.37^{f}	$9.14^{f\S}$	9.47^{f}	$9.19^{f\S}$	9.45^{f}	$9.20^{f\S}$	9.33^{f}	9.12	9.47^{f}	$9.24^{f\S}$	9.28	$9.55^{f\S}$
	±0.27	±0.24	±0.16	±0.25	±0.26	±0.23	±0.29	±0.34	±0.28	±0.32	±0.34	±0.37	±0.38
Bifidobacteria	6.85	7.67^{f}	$7.33^{\S}$	7.13	$7.69^{f\S}$	7.65^{f}	7.47^{f}	7.73^{f}	7.69^{f}	7.54^{f}	$7.83^{f\S}$	8.02^{f}	8.19^{f}
	±0.30	±0.17	±0.18	±0.16	±0.16	±0.17	±0.15	±0.17	±0.12	±0.28	±0.27	±0.28	±0.25
Bacteroides	7.98	7.86	7.90	7.95	$8.08^{\S}$	8.04	8.08	8.15	7.95	8.09	8.09	8.20^{f}	$7.89^{\S}$
	±0.10	±0.35	±0.12	±0.17	±0.20	±0.23	±0.22	±0.28	±0.19	±0.27	±0.28	±0.26	±0.19
Lactobacilli	7.15	7.64^{f}	7.43^{f}	7.59^{f}	7.45^{f}	7.57^{f}	7.32	7.51^{f}	7.69^{f}	7.47^{f}	$7.73^{f\S}$	7.55^{f}	7.57^{f}
	±0.28	±0.25	±0.14	±0.22	±0.16	±0.15	±0.17	±0.19	±0.17	±0.18	±0.17	±0.23	±0.14
Clostridia	7.55	7.49	7.65	7.59	$7.81^{f\S}$	8.03^{f}	8.00^{f}	8.05^{f}	$7.23^{f\S}$	7.68	7.48	7.63	$7.20^{f\S}$
	±0.13	±0.18	±0.24	±0.19	±0.20	±0.18	±0.24	±0.26	±0.21	±0.13	±0.23	±0.30	±0.18
E. coli	8.14	7.61^{f}	7.66^{f}	7.92	8.03	7.80^{f}	7.85^{f}	7.90^{f}	8.04	8.19	8.24	7.91^{f}	7.96
	±0.25	±0.16	±0.25	±0.17	±0.18	±0.12	±0.16	±0.19	±0.27	±0.26	±0.26	±0.20	±0.21
Eubacteria	8.06	7.79	7.84	8.57^{f}	8.69^{f}	8.60^{f}	$8.27^{\S}$	8.21	$7.75^{f\S}$	8.12	8.16	8.56^{f}	$8.28^{f\S}$
	±0.23	±0.22	±0.18	±0.27	±0.24	±0.22	±0.25	±0.24	±0.23	±0.22	±0.27	±0.27	±0.22

* Values are mean $\log_{10}$ cfu (ml batch culture)$^{-1}$ ± S.D. for three replicates after 5 and 24 h fermentation

† Mean count at time 0

f Significantly different from initial count ($P \leq 0.05$)

§ Significantly different from 10 h count ($P \leq 0.05$)

M, mellibiose; R, raffinose; FOS, Fructoligosaccharides; GMMa, GMM + *L. acidophilus*; GMMr, GMM + *L. reuteri*

Table 7. Differences in bacterial populations* between substrates after 24 h of fermentation

Group	GMMr				GMMa				GMM			
	M	R	FOS	GMM	GMMa	M	R	FOS	GMM	M	R	FOS
Total count	0.41^f	0.36^f	0.29^f	0.42^f	0.31^f	0.10	0.06	-0.02	0.11	-0.02	-0.07	-0.08
Bifidobacteria	0.86^f	0.50^f	0.72^f	0.50^f	0.35^f	0.50^f	0.14^f	0.37^f	0.14	0.36^f	0.00	0.22^f
Bacteroides	-0.01	-0.19^f	-0.19^f	-0.06	-0.20	0.19	0.01	0.01	0.14	0.05	-0.13	-0.13
Lactobacilli	0.14	0.11	0.24^f	-0.13	-0.16	0.30^f	0.28^f	0.41^f	0.03	0.27^f	0.24^f	0.37^f
Clostridia	-0.45^f	-0.60^f	-0.80^f	-0.03	-0.28^f	-0.17	-0.33^f	-0.52^f	0.25	-0.42^f	-0.58^f	-0.77^f
E. coli	0.30^f	-0.06	0.12	-0.08	-0.28^f	0.58^f	0.22^f	0.40^f	0.20^f	0.38^f	0.02	0.20^f
Eubacteria	0.44^f	-0.42^f	0.01	0.53^f	0.12	0.32^f	-0.54^f	-0.11	0.41^f	-0.09	-0.94^f	-0.51^f

* Values are differences of mean $\log_{10}$ cfu (ml batch culture)$^{-1}$ for three replicates after 24 h of fermentation

f Significantly different after 24h of fermentation ($P \leq 0.05$)

M, mellibiose; R, raffinose; FOS, Fructooligosaccharides; GMMa, GMM + *L. acidophilus*; GMMr, GMM + *L. reuteri*

Claims

1. A process for the production of an oligosaccharide, the process comprising:

combining an α-galactose substrate with a *Lactobacillus* α-galactosidase, under conditions which allow glycosyl transfer by the α-galactosidase and obtaining one or more oligosaccharides.

2. A process, as claimed in claim 1, wherein the α-galactose substrate is one or more of raffinose, stachyose or melibiose.

3. A process, as claimed in claim 1 or claim 2, wherein the *Lactobacillus* α-galactosidase is provided in a cell free extract.

4. A process as claimed in any one of claims 1-3, wherein the *Lactobacillus* α-galactosidase is provided by *Lactobacillus*.

5. A process, as claimed in any one of claims 1 to 4, wherein the oligosaccharide produced comprises one or more of glucose, galactose, fructose or a disaccharide.

6. A process, as claimed in any one of claims 1 to 5, wherein the *Lactobacillus* galactosidase is one or more of *Lactobacillus reuteri*, *ruminus*, *acidophilus*, *murinus* or *mucosae*.

7. A process, as claimed in any one of claims 1 to 6, wherein the oligosaccharide produced comprises one or more of the following fractions:

α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*; or

α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*

8. A novel oligosaccharide, obtainable by a process as claimed in any one of claims 1 to 7.

9. An oligosaccharide, which comprises one or more of the following fractions:

α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*; or
α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*

10. A composition which comprises an oligosaccharide as claimed in claim 8 or claim 8.

11. A composition as claimed in claim 10, which is in the form of a foodstuff.

12. An oligosaccharide as claimed in claim 8 or claim 9 or a composition as claimed in claim 10 or claim 11 for increasing beneficial bacteria in the gastrointestinal tract of an animal.

13. A synbiotic which comprises an oligosaccharide as claimed in claim 8 or claim 9 and a probiotic microorganism.

14. A synbiotic, as claimed in claim 13, wherein the probiotic microorganism is a *Lactobacillus*, such as *reuteri*, *ruminus*, *acidophilus*, *murinus*, or *mucosae*.

15. A synbiotic, as claimed in claim 13 or claim 14, which is the form of a foodstuff.

16. A synbiotic, as claimed in claim 15, wherein the foodstuff is a drink, a dry, wet or semi-moist foodstuff.

17. A process for producing a composition as claimed in claim 10 or claim 11 or a synbiotic as claimed in any one of claims 14 to 16, the process comprising combining the oligosaccharide with the other components and optionally heating and/or forming the combination into a product.

18. A method of obtaining a health benefit in an animal, the method comprising administering an oligosaccharide as claimed in claim 8 or claim 9, a composition as claimed in claim 10 or claim 11, or a synbiotic as claimed in any one of claims 13-16.

19. Use of an oligosaccharide as claimed in claim 8 or claim 9, in the manufacture of a composition for obtaining a health benefit in an animal.

Fig 1

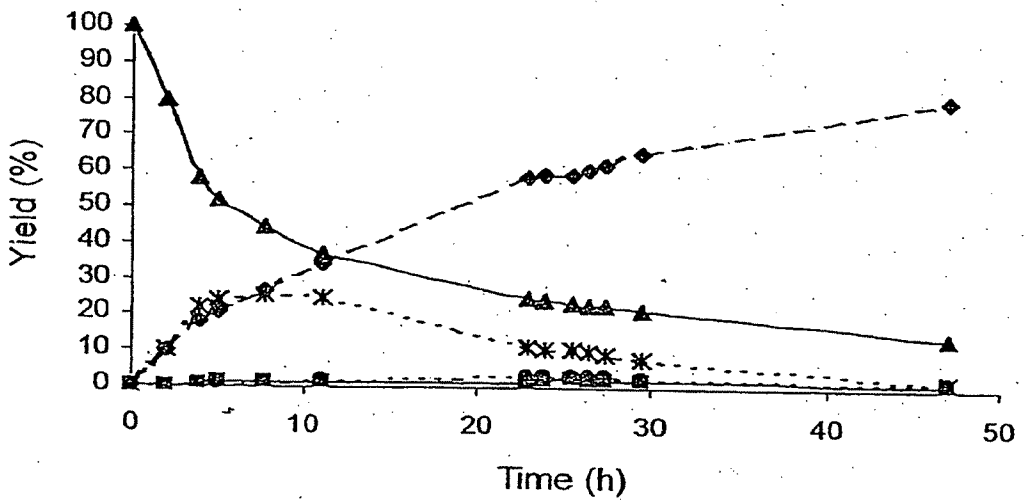


Fig 2

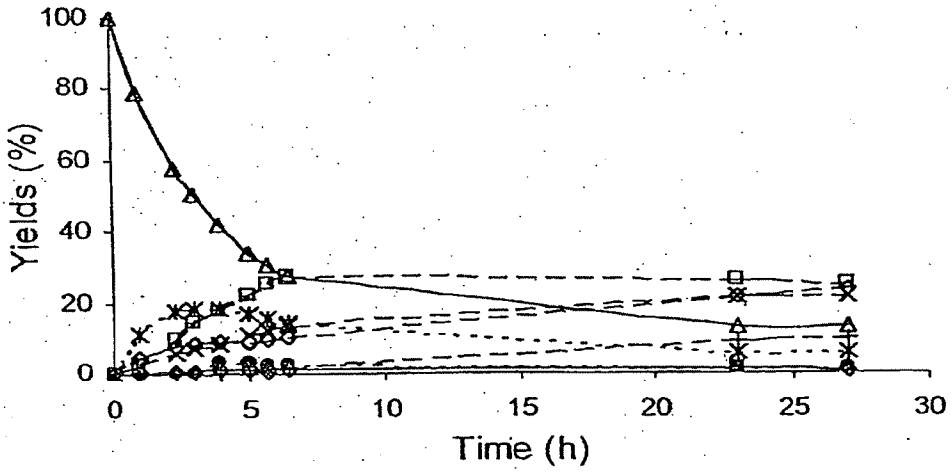


Fig 3

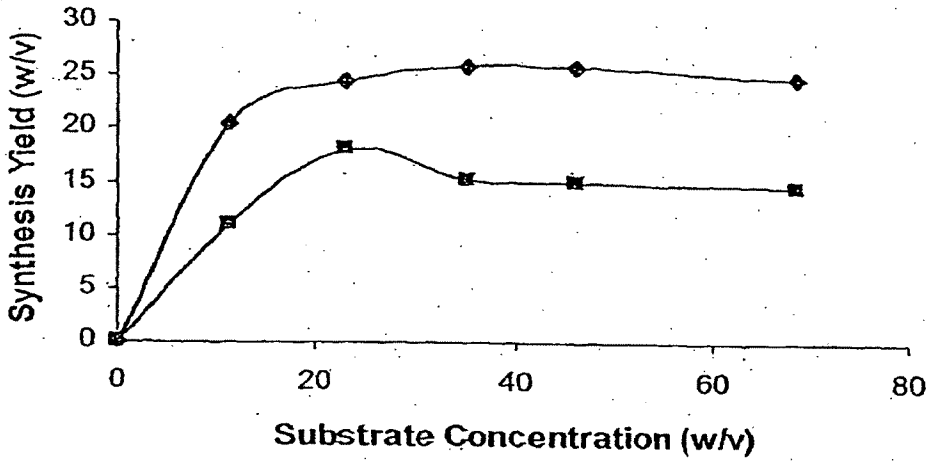

Synthesis Yield (w/v)
30
25
20
15
10
5
0
0
20
40
60
80
Substrate Concentration (w/v)

Fig 4

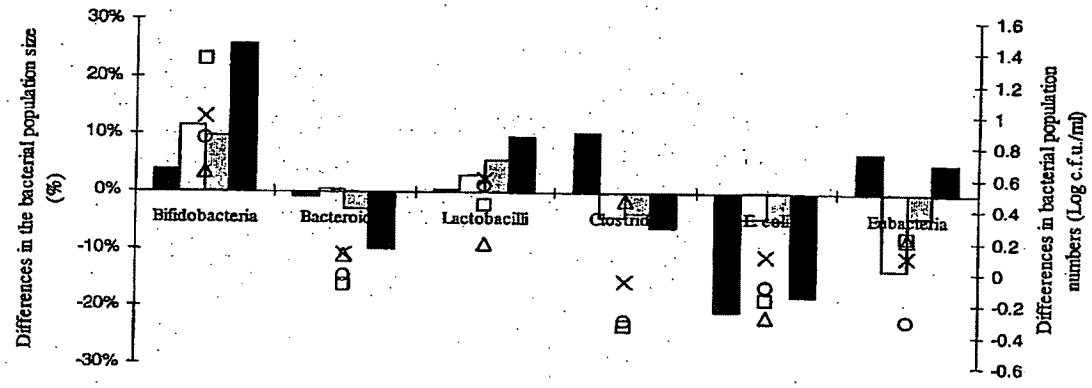
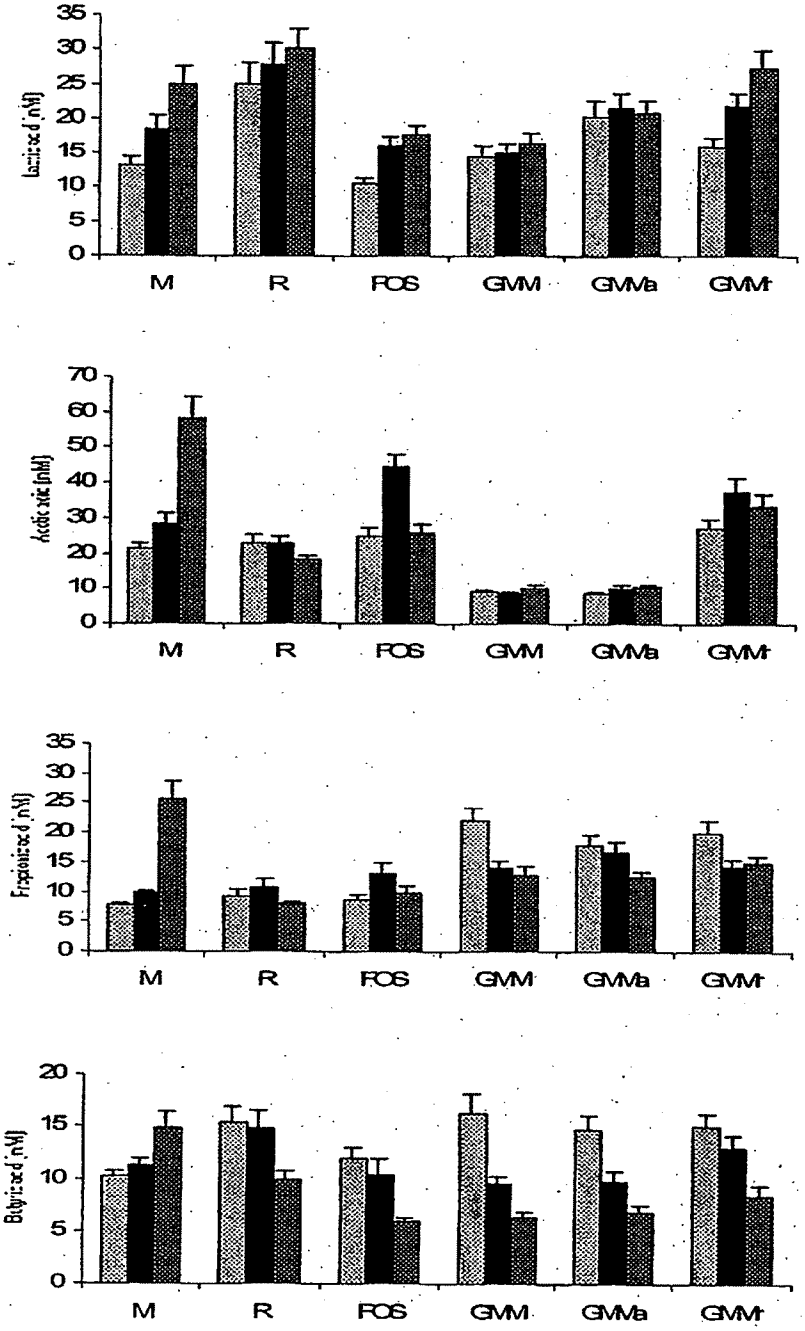

Differences in the bacterial population size (%)
30%
20%
10%
0%
-10%
-20%
-30%
Bifidobacteria
Bacteroid
Lactobacilli
Clostrid
E.coli
Enbacteria
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
Differences in bacterial population numbers (Log c.f.u./ml)

Fig 5


35
30
25
20
15
10
5
0
M
R
FOS
GMM
GMMa
GMMr
70
60
50
40
30
20
10
0
M
R
FOS
GMM
GMMa
GMMr
35
30
25
20
15
10
5
0
M
R
FOS
GMM
GMMa
GMMr
20
15
10
5
0
M
R
FOS
GMM
GMMa
GMMr

INTERNATIONAL SEARCH REPORT

ernational Application No
CT/GB2004/000627

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/14 C07H3/06 A23L1/09

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHULER R ET AL: "KINETIC PROPERTIES OF ALPHA-D GALACTOSIDASE EC-3.2.1.22 FROM LACTOBACILLUS-FERMENTI" ENZYME AND MICROBIAL TECHNOLOGY, vol. 7, no. 5, 1985, pages 207-211, XP002285613 ISSN: 0141-0229 Abstract; p. 208, col. 2, "Raffinose and stachyose hydrolysis" and "Soymilk hydrolysis"; Fig. 6 and 7. --- -/--	1-19

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 2004	05/07/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lopez García, F

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

ernational Application No
CT/GB2004/000627

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GARRO MARISA S ET AL: "Influence of carbohydrates on the alpha-galactosidase activity of Lactobacillus fermentum" CURRENT MICROBIOLOGY, vol. 33, no. 5, 1996, pages 302-305, XP002285614 ISSN: 0343-8651 Abstract; Fig .1;	1-19
X	VAN LAERE K M J ET AL: "Transglycosidase activity of Bifidobacterium adolescentis DSM 20083 alpha-galactosidase" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 52, no. 5, November 1999 (1999-11), pages 681-688, XP002285615 ISSN: 0175-7598 Abstract; p. 687, col. 1, 2nd full paragraph.	8-19
X	SUGAWARA S ET AL: "TRANSGALACTOSYLATION PRODUCTS FROM MELIBIOSE BY THE ALPHA GALACTOSIDASE OF ABSIDIA-CORYMBIFERA" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 54, no. 1, 1990, pages 211-214, XP001189675 ISSN: 0002-1369 p. 213, col. 1, 2nd line from bottom.	8-19
X,P	TZORTZIS G ET AL: "Synthesis of alpha-galactooligosaccharides with alpha-galactosidase from Lactobacillus reuteri of canine origin." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 63, no. 3, December 2003 (2003-12), pages 286-292, XP001181871 ISSN: 0175-7598 the whole document	1-19
X,P	TZORTZIS G ET AL: "In vitro evaluation of the fermentation properties of galactooligosaccharides synthesised by 'alpha!-galactosidase from Lactobacillus reuteri" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY 2004 GERMANY, vol. 64, no. 1, 2004, pages 106-111, XP002285616 ISSN: 0175-7598 the whole document	1-19

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/000627

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 18
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB2004 /000627

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Claims Nos.: 18

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy